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(54) Title: SEPARATION OF PLASMA COMPONENTS (57) Abstract A method for separating components from plasma, the method comprising (I) separating the plasma into a first and second component by causing the first component to move through a first electrophoretic separation membrane under the influence of an electric potential, the first component comprising an albumin/ α -1-antitrypsin pool and the second component comprising plasma containing components having a molecular mass greater than albumin; (II) treating the second component under the influence of an electric potential in the presence of a second electrophoretic separation membrane to form an immunoglobulins concentrate containing immunoglobulins substantially free from components having a molecular mass less than immunoglobulins; (III) treating the immunoglobulins concentrate under the influence of an electric potential in the presence of a third electrophoretic separation membrane to remove components having a molecular mass greater than immunoglobulins; and (IV) separating albumin and α -1-antitrypsin from the albumin/ α -1-antitrypsin pool by causing α -1-antitrypsin to move through a fourth electrophoretic separation membrane under the influence of an electric potential.		

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Separation of Plasma Components

Technical Field

The present invention relates to the separation of biomolecules from plasma, particularly human plasma.

5 Background Art

Human plasma contains approximately 3000 proteins with a variety of functions and potential therapeutic uses. Tight control of plasma available for blood fractionation means that the supply of important therapeutic agents like IgG is severely curtailed. This together with methodology which ends in
10 very low yields and takes three to five days contributes to the international shortfall of major plasma fractions.

The present inventors have found that rapid isolation times, high recoveries and high-resolution make Gradiflow™ technology a viable alternative purification technology to conventional Cohn precipitation and
15 column chromatography [Horvath SZ, Corthals GL, Wrigley CW and Margolis J. Multifunctional apparatus for electrokinetic processing of proteins. Electrophoresis 1994; 15: 968].

Albumin and IgG both have enormous importance in medicine and therefore are of considerable commercial value. Albumin alone has an
20 estimated annual global market value of \$US1.5 billion [SG Cowen, Perspectives Blood Transfusion Industry, October 1998, pp 54]. Conventional purification protocols are cumbersome and expensive with low yields and long processing times [Allen PC, Hill EA, Stokes AM in Plasma Proteins Analytical and Preparative Techniques, Blackwell Scientific
25 Publications, London 1977, pp. 182-189].

Albumin is the most abundant protein component (50 mg/mL) in human plasma and functions to maintain whole blood volume and oncotic pressure. Albumin also regulates the transport of protein, fatty acids, hormones and drugs [Allen PC, Hill EA, Stokes AM in Plasma Proteins
30 Analytical and Preparative Techniques, Blackwell Scientific Publications, London 1977, pp. 182-189]. Clinical uses include blood volume replacement during surgery, treatment of shock, serious burns and other medical emergencies and the stabilisation of other pharmaceutical products.

Albumin has a molecular mass of 67 kDa and an isoelectric point (pI)
35 of approximately 4.9. The protein comprises of a single subunit and is globular in shape [Andersson LO, in Blomback B, Lars HA (Eds), Plasma

Proteins, A Wiley Interscience Publication New York, 1979, pp 43-45]. Conventional purification schemes use the Cohn ethanol precipitation method and result in only 50% recovery.

Immunoglobulin G (IgG) is the most abundant of the immunoglobulins, representing almost 70% of the total immunoglobulin component in human serum. The concentration of IgG in normal plasma is approximately 10 mg/mL [Bennich H in Blomback B, Lars HA (Eds), Plasma Proteins, A Wiley Interscience Publication New York, 1979, pp 122]. The IgG plays an essential role in the immune response and have clinical uses including treatment of snake and spider bites, neurological disorders and IgG is commonly used in analytical or diagnostic kits.

The gamma-globulins have a molecular mass of approximately 150 kDa and consist of four chains, two of which are light and two of which are heavy [Bennich H in Blomback B, Lars HA (Eds), Plasma Proteins, A Wiley Interscience Publication New York, 1979, pp 122]. Immunoglobulins are traditionally isolated using Cohn ethanol precipitation or alternatively affinity chromatography [Allen PC, Hill EA, Stokes AM in Plasma Proteins Analytical and Preparative Techniques, Blackwell Scientific Publications, London 1977, pp. 178].

Alpha-1-antitrypsin is an acid glycoprotein of 54 kDa with an isoelectric point of 4.8 and is used in the treatment of hereditary emphysema [Allen PC, Hill EA, Stokes AM in Plasma Proteins Analytical and Preparative Techniques, Blackwell Scientific Publications, London 1977, pp. 210-211]. Conventional purification schemes utilise a combination of Cohn fractionation and column chromatography with the major difficulty being the removal of albumin from α -1-antitrypsin preparations [Allen PC, Hill EA, Stokes AM in Plasma Proteins Analytical and Preparative Techniques, Blackwell Scientific Publications, London 1977, pp. 212]. Current production schemes provide a yield of approximately 30% and much of this is contaminated with albumin. The present inventors have adapted Gradiflow™ to provide an alternative technique for producing highly pure α -1-antitrypsin with a yield of above 70%. This strategy also exemplifies Gradiflow™ technology's use in isolating protease inhibitors.

Gradiflow™ Technology

Gradiflow™ technology utilises molecular characteristics of size and charge to isolate protein [Horvath SZ, Corthals GL, Wrigley CW and Margolis J. Multifunctional apparatus for electrokinetic processing of proteins.

5 Electrophoresis 1994; 15: 968] with the resolution of two-dimensional electrophoresis and the throughput of preparative chromatography. Proteins exist as charged molecules above or below their isoelectric point (pI). In the Gradiflow™ the net charge on a macromolecule is controlled by the choice of buffer pH. The proteins are separated in an electric field by charge and/or
10 size differences. Some examples of Gradiflow™ technology may be found in US Patent Numbers 5039386 and 5650055, which US Patents are incorporated herein by reference.

The present inventors have found that the Gradiflow™ technology can be adapted to purify a number of different biomolecular components from
15 plasma. The present inventors have devised methodology for the rapid isolation of albumin, IgG and α -1-antitrypsin from a single volume of plasma in a four-phase process with high yield and low cost.

Disclosure of Invention

In a general aspect, the present invention relates to the sequential
20 separation of a number of biomolecules present in a plasma sample using four major separation phases or processes.

In a first aspect, the present invention relates to a method for separating components from plasma, the method comprising the phases:

- 25 (I) separating the plasma into a first and second component by causing the first component to move through a first electrophoretic separation membrane under the influence of an electric potential, the first component comprising an albumin/ α -1-antitrypsin pool and the second component comprising plasma containing components having a molecular mass greater than albumin;
- 30 (II) treating the second component under the influence of an electric potential in the presence of a second electrophoretic separation membrane to form an immunoglobulins concentrate containing immunoglobulins substantially free from components having a molecular mass less than immunoglobulins;
- 35 (III) treating the immunoglobulins concentrate under the influence of an electric potential in the presence of a third electrophoretic separation

membrane to remove components having a molecular mass greater than immunoglobulins; and

(IV) separating albumin and α -1-antitrypsin from the albumin/ α -1-antitrypsin pool by causing α -1-antitrypsin to move through a fourth electrophoretic separation membrane under the influence of an electric potential.

Phase I - Removal of albumin, α -1-antitrypsin and small contaminants

Preferably phase I comprises the steps:

- (a) placing the plasma in a first solvent stream, the first solvent stream being separated from a second solvent stream by a first electrophoretic separation membrane having a molecular mass cut-off less than the molecular mass of albumin and a restriction membrane having a molecular mass cut-off less than the first electrophoretic separation membrane;
- (b) selecting a buffer for the first solvent stream having a pH greater than the pI of albumin;
- (c) applying an electric potential between the two solvent streams causing movement of albumin and α -1-antitrypsin through the first electrophoretic membrane into the second solvent stream while biomolecules having a molecular mass greater than albumin and α -1-antitrypsin are substantially retained in the first solvent stream, or if entering the first electrophoresis membrane, being substantially prevented from passing through the first electrophoresis membrane, wherein biomolecules in the plasma having a molecular mass less than albumin and α -1-antitrypsin are caused to move through the first separation membrane and the restriction membranes to a waste collection;
- (d) optionally, periodically stopping and reversing the electric potential to cause movement of biomolecules having a molecular mass greater than albumin and α -1-antitrypsin having entered the first electrophoresis membrane to move back into the first solvent stream, wherein substantially not causing any albumin or α -1-antitrypsin that have entered the second solvent stream to re-enter first solvent stream;
- (e) maintaining steps (c) and optionally (d) until the desired amount of albumin and α -1-antitrypsin have been collected as an albumin/ α -1-antitrypsin pool and biomolecules having a molecular mass less than albumin and α -1-antitrypsin have been removed from the first solvent stream to form a treated plasma;

Phase II - Removal of large contaminants

Preferably phase II comprises the steps:

- 5 (f) placing the treated plasma in a third solvent stream, the third solvent stream being separated from a fourth solvent stream by a second electrophoretic separation membrane having a molecular mass cut-off less than the molecular mass of immunoglobulins;
- (g) selecting a buffer for the third solvent stream having a pH above neutral;
- 10 (h) applying an electric potential between the third and fourth solvent streams causing movement of biomolecules having a molecular mass less than that of immunoglobulins in the treated plasma through the second electrophoretic separation membrane into the fourth solvent stream while immunoglobulins and other biomolecules having a molecular mass greater than immunoglobulins are substantially retained in the third solvent stream,
- 15 or if entering the second electrophoresis separation membrane, being substantially prevented from passing through the second electrophoresis separation membrane;
- (i) optionally, periodically stopping and reversing the electric potential to cause movement of immunoglobulins and other biomolecules having a
- 20 molecular mass greater than immunoglobulins having entered the second electrophoresis separation membrane to move back into the third solvent stream, wherein substantially not causing any biomolecules having a molecular mass less than immunoglobulins that have entered the fourth solvent stream to re-enter third solvent stream;
- 25 (j) maintaining steps (h) and optional (i) until the desired amount of biomolecules having a molecular mass less than immunoglobulins have been removed from the third upstream to form an immunoglobulins concentrate;
- (k) removing the biomolecules from the fourth solvent stream;

Phase III - Separation of immunoglobulins

30 Preferably phase III comprises the steps:

- (l) replacing the second electrophoretic separation membrane with a third electrophoretic separation membrane having a molecular mass cut-off greater than the molecular mass of immunoglobulins;
- 35 (m) selecting a buffer for the immunoglobulins concentrate having a pH below neutral;

(n) applying an electric potential between the immunoglobulins concentrate in the third solvent stream and a fresh fourth solvent stream causing movement of immunoglobulins in the immunoglobulins concentrate in the third solvent stream through the third electrophoretic separation membrane into the fresh fourth solvent stream while biomolecules having a molecular mass greater than immunoglobulins are substantially retained in the third solvent stream, or if entering the third electrophoresis separation membrane, being substantially prevented from passing through the third electrophoresis separation membrane;

(o) optionally, periodically stopping and reversing the electric potential to cause movement of biomolecules having a molecular mass greater than immunoglobulins having entered the third electrophoresis membrane to move back into the treated third solvent stream, wherein substantially not causing any immunoglobulins that has entered the fresh fourth solvent stream to re-enter treated third solvent stream;

(p) maintaining steps (n) and optional (o) until the desired amount of immunoglobulins have been moved to the fresh fourth downstream;

Phase IV - Separation of albumin from α -1-antitrypsin

Preferably phase IV comprises the steps:

(q) placing the albumin/ α -1-antitrypsin concentrate in a fifth solvent stream, the fifth solvent stream being separated from a sixth solvent stream by a fourth electrophoretic separation membrane having a molecular mass cut-off less than the molecular mass of albumin;

(r) selecting a buffer for the fifth solvent stream having a pH greater than neutral;

(s) applying an electric potential between the fifth and sixth solvent streams causing movement of α -1-antitrypsin through the fourth electrophoresis separation membrane into the sixth solvent stream while albumin is substantially retained in the fifth solvent stream, or if entering the fourth electrophoresis separation membrane, being substantially prevented from passing through the fourth electrophoresis separation membrane;

(t) optionally, periodically stopping and reversing the electric potential to cause movement of albumin having entered the fourth electrophoresis separation membrane to move back into the fifth solvent stream, wherein substantially not causing any α -1-antitrypsin that has entered the sixth solvent stream to re-enter the fifth solvent stream; and

(u) maintaining steps (s) and optionally (t) until the desired amount of albumin remains in the fifth solvent stream and the desired amount of α -1-antitrypsin has have been removed to the sixth solvent stream.

5 As the present invention is directed to the sequential separation of a number of components from plasma, phase IV comprising steps (q) to (u) can be carried out before phase II comprising steps (f) to (p). Phase I, the initial steps (a) to (e) produces two products, namely albumin/ α -1-antitrypsin pool in the downstream and treated plasma in the upstream. Each of these two products are processed further to produce isolated immunoglobulins,
10 albumin and α -1-antitrypsin.

Preferably, albumin, immunoglobulins and α -1-antitrypsin are separated from a pooled human plasma sample.

The present invention is particularly suited for the separation of immunoglobulin G (IgG).

15 Preferably, the first electrophoresis separation membrane of step (a) has molecular mass cut-off of about 75 kDa and the restriction membrane has a molecular mass cut off of about 50 kDa. Additional membranes may be positioned before, between or after the separation and restriction membranes to further enhance the separation method.

20 Preferably, the buffer in step (b) has a pH of about 9. A Tris-borate buffer has been found to be particularly suitable for this separation. It will be appreciated, however, that other buffers having a suitable pH range would also be suitable.

25 Preferably the second electrophoresis separation membrane of step (f) has a molecular mass cut-off of about 200 kDa. The third electrophoresis separation membrane of step (l) preferably has a molecular mass cut-off of about 500 kDa.

30 Preferably, the buffer of the third solvent stream in step (g) has a pH of about 9 and the buffer of the treated third solvent stream of step (m) has a pH of less than about 5, more preferably about pH 4.6.

Preferably, the fourth electrophoresis separation membrane of step (q) has molecular mass cut-off of about 50 kDa.

35 Preferably, the buffer in step (r) has a pH of about 8.0. A Tris-borate buffer has been found to be particularly suitable for this separation. It will be appreciated, however, that other buffers having a suitable pH range would also be suitable.

A potential of 250 volts has been found to be suitable for the separation process. Other voltages, higher or lower, would also be suitable for the present invention depending on the separation membrane(s) used, volume of plasma or treated materials to be processed and the speed of separation required.

Preferably, the first and second solvent streams form part of a first Gradiflow™ apparatus and the third and fourth solvent streams form part of a second Gradiflow™ apparatus.

The purified albumin may be concentrated using a Gradiflow™ system incorporating an electrophoresis separation membrane having a molecular mass cut-off less than the molecular mass of albumin in a pH of greater than 8, preferably about pH 8.4.

The benefits of the method according to the first aspect of the present invention are the possibility of scale-up without adversely altering the properties of the plasma components being separated.

The method according to the present invention results in yields of albumin, immunoglobulins, preferably IgG, and α -1-antitrypsin from plasma of at least 70% with a purity of at least 90% from pooled samples of plasma.

The method according to the present invention results in substantially purified or isolated albumin, immunoglobulins, preferably IgG, and α -1-antitrypsin from plasma in less than 1 day, preferably in less than 12 hours, and more preferably in less than 6 hours. The speed of separation and purity of the final components (albumin, immunoglobulins, preferably IgG, and α -1-antitrypsin) provides a great advance over the prior art methods. Not only does the method allow the processing of one sample of plasma to obtain three major components (albumin, immunoglobulins, preferably IgG, and α -1-antitrypsin), the method is fast and extremely efficient.

In a second aspect, the present invention relates to use of Gradiflow™ in the purification and/or separation of albumin, immunoglobulins, preferably IgG, and α -1-antitrypsin from plasma.

In a third aspect, the present invention relates to albumin, immunoglobulins, preferably IgG, and α -1-antitrypsin purified by the method according to the first aspect of the present invention.

In a fourth aspect, the present invention relates to use of albumin, immunoglobulins, preferably IgG, and α -1-antitrypsin according to the third aspect of the present invention in medical and veterinary applications.

The purification of individual components of plasma is an important illustration of the power of Gradiflow™ in isolating products from complex biological solutions.

5 Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

10 In order that the present invention may be more clearly understood preferred forms will be described with reference to the following drawings.

Brief Description of Drawings

15 Figure 1, 8-16% non-reduced sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) gel. Albumin was isolated from plasma (lane 2) by its migration through the 75 kDa separation membrane into the downstream (lanes 5-10). Smaller molecular weight contaminants dissipated through the 50 kDa restriction membrane. Albumin was harvested at 30 minute intervals for a total of 180 minutes. Residual plasma proteins were retained in the upstream (lane 3) for subsequent IgG purification.

20 Figure 2, size exclusion high performance liquid chromatography (HPLC). Albumin prepared using Gradiflow™ technology was compared with a commercial therapeutic preparation. HPLC was performed using a Shimadzu SCL-10A VP HPLC system in combination with a ZORBAX GF 250 4.6 x 250 mm analytical column. Samples were run at pH 7, 100 mM phosphate buffer containing 200 mM NaCl.

25 Figure 3, 4-20% reduced SDS PAGE gel. Residual plasma proteins from the albumin isolation (lane 3) were further fractionated in a two-phase process, the first of which removes contaminants of less than 200 kDa. The second phase transferred the IgG component from the upstream to the downstream where it was concentrated (lanes 3-6).

30 Figure 4, Western analysis of a 4-20% reduced SDS PAGE gel. The product from phase 2 of the purification was Western blotted and incubated with DAKO anti-immunoglobulin antibody. The stained bands indicate that multiple immunoglobulin families were isolated from plasma. Further processing of the sample would allow individual families to be purified.

35 Figure 5, Non-reduced SDS PAGE phoretix. Gradiflow™ purified IgG preparation was compared with a commercial therapeutic preparation.

Figure 6, 8-16% non-reduced SDS PAGE. Alpha-1-antitrypsin was isolated from Gradiflow™ purified albumin (lane 2) by its migration through the 50 kDa separation membrane into the downstream (lanes 7-9). Alpha-1-antitrypsin was harvested at 60 minute intervals for a total of 180 minutes. Residual albumin was retained in the upstream (lanes 3-5).

Figure 7, Western analysis of 8-16% non-reduced SDS PAGE. Alpha-1-antitrypsin was isolated from Gradiflow™ purified albumin (lane 1) by its migration through the 50 kDa separation membrane into the downstream (lanes 6-8).

Figure 8, α -1-antitrypsin functional analysis. Alpha-1-antitrypsin biological activity was investigated using a chromogenic elastase inhibition assay. Gradiflow™ α -1-antitrypsin fractions showed activity, in contrast to the residual albumin product.

Modes for Carrying Out the Invention

MATERIALS AND METHODS

Reagents

All chemicals unless otherwise stated were provided by Sigma (St Louis, MO). Boric Acid was obtained from ICN (Costa Mesa, CA). Methanol was provided by Merck (Kilsyth, Vic).

Tris-Borate (TB) Running Buffer:

6.5 g trisma base, 1.275 g boric acid, deionised H₂O to 1 L, pH 9.0.

Tris-Borate (TB) Running Buffer:

7.74 g trisma base, 11.87 g boric acid, deionised H₂O to 1 L, pH 8.0.

GABA-Acetic Acid Running Buffer:

3.165 g GABA, 1.08 mL acetic Acid, deionised H₂O to 1 L, pH 4.6.

Gradipore Glycine Sample Buffer:

10% (w/v) SDS, 2.0 mL glycerol, 0.1% (w/v) bromophenol blue, 0.5 M tris-HCl (pH 6.8), deionised H₂O to 10 mL.

Dithiothreitol (DTT):

3 mg DTT per 1 mL methanol.

SDS Glycine Running Buffer:

2.9 g tris base, 14.4 g glycine, 1 g SDS, deionised H₂O to 1 L, pH 8.3.

Towbin buffer:

25 mM tris, 192 mM glycine, 20% methanol, deionised H₂O, pH 8.3.

Phosphate Buffered Saline (PBS):

9 g NaCl, 0.2 g KH₂PO₄, 2.9 g Na₂HPO₄, 2 g KCl, deionised H₂O to 1 L, pH 7.2.

4-Chloro-1-naphthol (4CN): 3 mg 4CN per mL of methanol.

Gradipure™:

Coomassie Brilliant Blue < 1% w/v, ammonium sulphate ~ 10% w/v,
orthophosphoric acid ~ 1% v/v, methanol ~ 20% v/v.

5 Albumin Isolation

Pooled normal plasma was diluted one part in three with Tris-borate (TB) running buffer, pH 9.0 and placed in the upstream of Gradiflow™ apparatus. Albumin was isolated from platelet free plasma in a one-phase process using the charge of albumin at a pH above its isoelectric point and its
10 molecular weight. A separation cartridge with a 75 kDa cut-off separation membrane was placed between two 50 kDa cut-off restriction membranes. Upon application of 250 volts across the separation unit, albumin was removed from higher molecular weight contaminants by its migration through the separation membrane whilst smaller molecular weight
15 contaminants dissipated through the 50 kDa cut-off restriction membrane. Albumin was harvested at 30 minute intervals for a total of 180 minutes.

The purity of the preparation was determined using SDS PAGE (Gradipore Tris-Glycine 8-16% gradient gels) and size exclusion HPLC.

A Bromocresol green kit (BCG) was supplied by Trace Scientific
20 (Clayton, Melbourne, Australia) and was used to determine albumin concentration throughout the isolation procedure [Doumas BT, Watson WA, Briggs HG. Albumin standards and the measurement of serum albumin with bromocresol green. Clin. Chimm. Acta, 31 (1971) p. 87]. Analysis was performed according to manufacturer's instructions.

25 Immunoglobulin (IgG) Isolation

The upstream residual from the albumin isolation was further processed using a 200 kDa cut-off separation cartridge together with a TB running buffer, pH 9.0. A potential of 250 volts was applied across the separation unit for 1 hour. A membrane of this size, in combination with the
30 low charge to mass ratio of IgG at pH 9, restricts IgG migration whilst allowing smaller molecular weight contaminants to pass through the membrane, leaving IgG and higher molecular weight contaminants in the upstream. A second purification phase was carried out at pH 4.6 using a 500 kDa cut-off separation membrane for 2 hours. IgG migrated through the
35 separation membrane when 250 volts reversed polarity potential was applied, leaving other high molecular weight contaminants upstream.

Western blot analysis was carried out as described by Towbin et al (1979) [Towbin H, Staehelin T and Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 1979; 76: 4350] on selected SDS gels. Blotting filter paper and nitrocellulose blotting membrane were pre-soaked in Towbin buffer for 60 minutes. Protein transfer was performed in semi-dry blotting apparatus (Macquarie University, Sydney, Australia) at 12V for 90 minutes. The membrane was washed with PBS for 5 minutes, blocked with 1% skim milk in PBS for 10 minutes. The membrane was stained with 20 μ L rabbit anti-human IgA, IgG, IgM, Kappa, Lambda conjugated to horseradish peroxidase (HRP) in 10 mL 1% skim milk solution for 60 minutes. The stain was developed with 4CN diluted one part in five in PBS to a volume of 10 mL and 10 μ L H₂O₂. Development of the blot occurred within 30 minutes.

α -1-Antitrypsin Isolation

The downstream product of the albumin purification was further processed using a 50 kDA cut-off separation membrane together with a TB running buffer, pH 8.0. A potential of 250 volts was applied across the separation unit for 3 hours. The α -1-antitrypsin was transferred to the downstream where it was harvested hourly. Further purified albumin remained upstream. Samples were analysed for purity using SDS PAGE.

Western blot analysis was carried out as described by Towbin et al (1979) [Towbin H, Staehelin T and Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 1979; 76: 4350] on selected SDS gels. Blotting filter paper and nitrocellulose blotting membrane were pre-soaked in Towbin buffer for 60 minutes. Protein transfer was performed in semi-dry blotting apparatus (Biorad) at 15V for 60 minutes. The membrane was washed with PBS for 5 minutes, blocked with 1% skim milk in PBS/0.1% Tween 20 (v/v) for 10 minutes. The membrane was incubated with 10 μ L monoclonal anti-human α -1-antitrypsin (Biodesign, Clone number 1102) in 10 mL 1% skim milk solution for 60 minutes. The membrane was then tagged with DAKO rabbit anti-mouse HRP conjugate in 1% skim milk solution for 60 minutes. The membrane was developed with 4CN diluted one part in five in PBS to a volume of 10 mL and 10 μ L H₂O₂. Development of the blot occurred within 30 minutes.

Alpha-1-antitrypsin recovery was measured using a Behring Nephelometer 100 Analyzer (Dade Behring, Marburg, Germany). Assays were performed using rabbit anti-human α -1-antitrypsin nephelometry reagent (Dade Behring OSAZ 15) and carried out according to manufacturer's instruction.

Alpha-1-antitrypsin functionality was investigated using chromogenic elastase neutralisation assay. Elastase was diluted 1:1, 1:5, 1:10, 1:20, 1:40, 1:80, 1:160, 1:320 with pH 8.0 buffer (N.B. the stock elastase from Sigma was 32 U/ml). Fifty μ l of each elastase dilution was added to 50 μ l of α -1-antitrypsin sample, and shaken for 15 minutes. A control set of samples was also prepared in which each elastase dilution was combined with an equal volume of running buffer. Twenty μ l of each mixture was pipetted into wells of a flat bottom microtitre plate, and 150 μ l of the Pefa-ELA substrate (Pentapharm Basel, Switzerland) freshly diluted 1:100 with pH 8.0 buffer added. (N.B. each vial is reconstituted with 1 ml of DMSO and stored at +4°C). Colour development was monitored at 37°C in a plate reader (Versamax, Molecular Devices) for 2 hours at a wavelength of 405nm. The kinetic analysis was made by calculating the Vmax over 20 points for each well. Plots of Vmax against elastase concentration were made on a log-log scale. The linear section of the plot was extrapolated to the x-axis to derive the concentration of antitrypsin in terms of elastase neutralisation units.

Albumin contamination was investigated using a Bromocresol green kit (BCG) supplied by Trace Scientific (Clayton, Melbourne, Australia) [Doumas BT, Watson WA, Briggs HG. Albumin standards and the measurement of serum albumin with bromocresol green. Clin. Chimm. Acta, 31 (1971) p. 87]. Analysis was performed according to manufacturer's instructions.

Anti-thrombin III contamination was investigated using an ELISA assay. One hundred μ L Heparin (1.5 mg/mL) was bound to a flat-bottomed microtitre plate overnight. The plate was washed three time with 250 μ L PBS/Tween 20 (0.1% v/v) before application of 50 μ L anti-thrombin III standards (Sigma, St Louis, MO), 50 μ L upstream and 50 μ L downstream samples (1:10 PBS/Tween 20). The plate was incubated at room temperature for 1 hour and washed, again with PBS/Tween 20. Fifty μ L DAKO rabbit anti-human anti-thrombin III (1:1000 PBS/Tween 20) was applied and the plate incubated for a further 1 hour. The plate was then washed and 50 μ L DAKO goat anti rabbit HRP conjugate applied. Washing of the plate and

development using 100 μ L o-toluidine followed incubation of the plate for 1 hour. Development was stopped using 50 μ L 3M HCl. The plate was read at 450nm and the samples compared to the generated standard curve.

5 SDS PAGE [Laemmli U. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227: 680-685] was performed using Tris-glycine-SDS running buffer. SDS PAGE samples were prepared using 40 μ L Gradipore glycine sample buffer, 10 μ L DTT, 50 μ L sample and were boiled for 5 minutes. SDS PAGE was run at 150 Volts for 90 minutes.

10 All SDS PAGE gels were stained with Gradipure™ (Gradipore, Sydney, Australia).

HPLC was performed using a Shimadzu SCL-10A VP HPLC system in combination with a ZORBAX GF 250 4.6 x 250 mm analytical column. Samples were run at pH 7, 100 mM phosphate buffer containing 200 mM NaCl.

15 RESULTS

Albumin Isolation

The one step purification procedure was successful in producing albumin that was greater than 95% pure with a recovery of 72%. The SDS
20 PAGE in Figure 1 illustrates the purification procedure. Albumin was isolated from plasma (lane 2) by its migration through the 75 kDa separation membrane into the downstream (lanes 5-10). Smaller molecular weight contaminants dissipated through the 50 kDa restriction membrane. Albumin was harvested at 30 minute intervals for a total of 180 minutes. Residual
25 plasma proteins were retained in the upstream (lane 3) for subsequent IgG purification albumin was isolated from plasma with single peak purity and compared with a commercially available therapeutic product (Figure 2). Albumin prepared using Gradiflow™ technology was compared with a commercial therapeutic preparation. HPLC was performed using a Shimadzu
30 SCL-10A VP HPLC system in combination with a ZORBAX GF 250 4.6 x 250 mm analytical column. Samples were run at pH 7, 100 mM phosphate buffer containing 200 mM NaCl. The entire purification phase took only 3 hours in duration, illustrating the rapidity of the method. The processing of the albumin preparation in the isolation of α -1-antitrypsin further increased the
35 purity of the Gradiflow albumin product.

Immunoglobulin (IgG) Isolation

The processing of the residual upstream from the albumin separation decreased the waste of important plasma components through the process. Furthermore, the running time of the IgG isolation was decreased due to the removal of albumin in the first purification phase. Figures 3 and 4 show reduced SDS PAGE and a corresponding Western blot analyses illustrating the presence of the characteristic heavy and light chains of IgG. Residual plasma proteins from the albumin isolation (lane 3) were further fractionated in a two-phase process, the first of which removes contaminants of less than 200 kDa. The second phase transferred the IgG component from the upstream to the downstream where it was concentrated (lanes 3-6). The product from phase 2 of the purification was Western blotted and incubated with DAKO anti-immunoglobulin antibody. The stained bands indicate that multiple immunoglobulin families were isolated from plasma (Figure 4). The purity of the immunoglobulin product was determined as 95-100% (Figure 5) using PAGE phoretix. Gradiflow™ purified IgG preparation was compared with a commercial therapeutic preparation and showed similar purity and characteristics.

Further processing of the product would allow specific immunoglobulin families to be isolated in the process, increasing the purity of the specific groups. Immunoglobulin yield was determined using HPLC and calculated to be greater than 75%.

α -1-Antitrypsin Isolation

α -1-Antitrypsin was purified from the Gradiflow™ purified albumin preparation with a recovery of 73%. Figure 6 illustrates the purity of α -1-antitrypsin obtainable using the present invention and in combination with the retention of biological activity provides a demonstration of the ability to purify functional proteins using Gradiflow™ technology. Alpha-1-antitrypsin was isolated from Gradiflow™ purified albumin (lane 2) by its migration through the 50 kDa separation membrane into the downstream (lanes 7-9). Alpha-1-antitrypsin was harvested at 60 minute intervals for a total of 180 minutes. Residual albumin was retained in the upstream (lanes 3-5). The removal of α -1-antitrypsin from the albumin preparation resulted in higher purity albumin and also minimised the time of isolation of α -1-antitrypsin. The other advantage of processing Gradiflow™ fractions was the reduction in waste of important plasma proteins. The retention of α -1-antitrypsin activity

was demonstrated by its ability to inhibit elastase activity. No detectable activity remained in the albumin preparation.

Figure 7 shows Western analysis of 8-16% non-reduced SDS PAGE. Alpha-1-antitrypsin was isolated from Gradiflow™ purified albumin (lane 1) by its migration through the 50 kDa separation membrane into the downstream (lanes 6-8). Figure 8 shows α -1-antitrypsin functional analysis where α -1-antitrypsin biological activity was investigated using a chromogenic elastase inhibition assay. Gradiflow™ purified α -1-antitrypsin fractions showed activity, in contrast to the residual albumin product.

Albumin contamination of the active α -1-antitrypsin product was demonstrated to be at most 0.061 mg/mL. The need for extra albumin decontamination steps using conventional isolation techniques is minimal. The absence of anti-thrombin III from the α -1-antitrypsin preparation further illustrated the exceptional resolution of Gradiflow technology.

Simultaneous Separations

Current methods for plasma protein separation involve the use of Cohn fractionation, which can take from 3-5 days to separate proteins into their purified form. Using the Gradiflow™ technology it is possible to substantially reduce the separation time from three days to three hours. By linking several Gradiflow™ machines in succession it is possible to simultaneously separate several proteins to single band purity from plasma in the same three hour period required to separate each individual protein. By linking several Gradiflow™ apparatus together in series, the plasma can be separated into several different fractions with different purified proteins being collected into separate streams. Linear scalability of the Gradiflow™ allows the separation of multiple numbers of proteins in a single three hour period rather than a minimum of two to three hours per protein if only one machine is used.

Plasma, suitably diluted, is placed into the first stream in a first apparatus and separated through a 200 kDa separation membrane. The selection of the separation membrane in this step has two functions. This membrane pore size allows all the albumin and α -1-antitrypsin to pass downstream where the two proteins can be further purified. Furthermore, this membrane allows all protein contaminants under 200 kDa to be removed from the immunoglobulins and other high molecular mass components which are retained in the first stream.

A second Gradiflow™ apparatus containing an 80 kDa separation membrane is used to process the downstream from the first apparatus. This membrane allows only albumin and α -1-antitrypsin to pass through into a third downstream whilst all larger contaminants are held in the second stream. A third apparatus which contains a 40 kDa separation membrane is connected to the second apparatus to process the third downstream containing albumin and α -1-antitrypsin. The selection of this membrane prevents the transfer of albumin from the third stream but allows the α -1-antitrypsin to pass through where it is collected in a fourth stream. Following this separation, substantially pure albumin remains in the third stream and substantially pure α -1-antitrypsin is collected in the fourth stream.

Once albumin and α -1-antitrypsin have been separated into their separate streams, third and fourth consecutively, IgG can then be separated from the treated first stream. This is achieved by disconnecting the first apparatus from the second and third apparatus and changing the pH of the buffer. A pH 4.6 GABA/Acetic acid buffer is suitable and the potential is reversed as per the protocol for a normal second phase IgG separation.

All three proteins, albumin, α -1-antitrypsin, and IgG, can be separated to single band purity with over 80% yield using the coupled apparatus. Both albumin and α -1-antitrypsin take about three hours to purify whilst IgG takes several hours longer due to the need to separate the three apparatus once the albumin and α -1-antitrypsin have been separated.

CONCLUSIONS

A method to rapidly purify albumin, IgG and α -1-antitrypsin from a single volume of plasma has been established. The minimisation of waste and the removal of various processing steps including ethanol precipitation and ultra-filtration demonstrate the potential of Gradiflow™ technology in the large-scale purification of blood proteins. Optimisation of the process would allow the removal of specific families and even species of the immunoglobulins. Further processing of Gradiflow™ waste fractions may allow the removal of many other important plasma molecules, providing a means by which to maximise the potential of plasma as a biopharmaceutical source. The high specificity of Gradiflow™ technology could allow specific molecules to be targeted and removed by applying suitable strategies.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to
5 be considered in all respects as illustrative and not restrictive.

CLAIMS:

1. A method for separating components from plasma, the method comprising the phases:
 - 5 (I) separating the plasma into a first and second component by causing the first component to move through a first electrophoretic separation membrane under the influence of an electric potential, the first component comprising an albumin/ α -1-antitrypsin pool and the second component comprising plasma containing components having a molecular mass greater than albumin;
 - 10 (II) treating the second component under the influence of an electric potential in the presence of a second electrophoretic separation membrane to form an immunoglobulins concentrate containing immunoglobulins substantially free from components having a molecular mass less than immunoglobulins;
 - 15 (III) treating the immunoglobulins concentrate under the influence of an electric potential in the presence of a third electrophoretic separation membrane to remove components having a molecular mass greater than immunoglobulins; and
 - 20 (IV) separating albumin and α -1-antitrypsin from the albumin/ α -1-antitrypsin pool by causing α -1-antitrypsin to move through a fourth electrophoretic separation membrane under the influence of an electric potential.
2. The method according to claim 1 wherein phase I comprises the steps:
 - 25 (a) placing the plasma in a first solvent stream, the first solvent stream being separated from a second solvent stream by a first electrophoretic separation membrane having a molecular mass cut-off less than the molecular mass of albumin and a restriction membrane having a molecular mass cut-off less than the first electrophoretic separation membrane;
 - 30 (b) selecting a buffer for the first solvent stream having a pH greater than the pI of albumin;
 - (c) applying an electric potential between the two solvent streams causing movement of albumin and α -1-antitrypsin through the first electrophoretic membrane into the second solvent stream while biomolecules having a molecular mass greater than albumin and α -1-antitrypsin are substantially
35 retained in the first solvent stream, or if entering the first electrophoresis membrane, being substantially prevented from passing through the first

electrophoresis membrane, wherein biomolecules in the plasma having a molecular mass less than albumin and α -1-antitrypsin are caused to move through the first separation membrane and the restriction membranes to a waste collection;

5 (d) optionally, periodically stopping and reversing the electric potential to cause movement of biomolecules having a molecular mass greater than albumin and α -1-antitrypsin having entered the first electrophoresis membrane to move back into the first solvent stream, wherein substantially not causing any albumin or α -1-antitrypsin that have entered the second
10 solvent stream to re-enter first solvent stream;

(e) maintaining steps (c) and optionally (d) until the desired amount of albumin and α -1-antitrypsin have been collected as an albumin/ α -1-antitrypsin pool and biomolecules having a molecular mass less than albumin and α -1-antitrypsin have been removed from the first solvent stream
15 to form a treated plasma;

3. The method according to claim 1 wherein phase II comprises the steps:

(f) placing the treated plasma in a third solvent stream, the third solvent stream being separated from a fourth solvent stream by a second electrophoretic separation membrane having a molecular mass cut-off less
20 than the molecular mass of immunoglobulins;

(g) selecting a buffer for the third solvent stream having a pH above neutral;

(h) applying an electric potential between the third and fourth solvent streams causing movement of biomolecules having a molecular mass less
25 that that of immunoglobulins in the treated plasma through the second electrophoretic separation membrane into the fourth solvent stream while immunoglobulins and other biomolecules having a molecular mass greater than immunoglobulins are substantially retained in the third solvent stream, or if entering the second electrophoresis separation membrane, being
30 substantially prevented from passing through the second electrophoresis separation membrane;

(i) optionally, periodically stopping and reversing the electric potential to cause movement of immunoglobulins and other biomolecules having a molecular mass greater than immunoglobulins having entered the second
35 electrophoresis separation membrane to move back into the third solvent stream, wherein substantially not causing any biomolecules having a

molecular mass less than immunoglobulins that have entered the fourth solvent stream to re-enter third solvent stream;

(j) maintaining steps (h) and optional (i) until the desired amount of biomolecules having a molecular mass less than immunoglobulins have been removed from the third upstream to form an immunoglobulins concentrate;

(k) removing the biomolecules from the fourth solvent stream;

4. The method according to claim 1 wherein phase III comprises the steps:

(l) replacing the second electrophoretic separation membrane with a third electrophoretic separation membrane having a molecular mass cut-off greater than the molecular mass of immunoglobulins;

(m) selecting a buffer for the immunoglobulins concentrate having a pH below neutral;

(n) applying an electric potential between the immunoglobulins concentrate in the third solvent stream and a fresh fourth solvent stream causing movement of immunoglobulins in the immunoglobulins concentrate in the third solvent stream through the third electrophoretic separation membrane into the fresh fourth solvent stream while biomolecules having a molecular mass greater than immunoglobulins are substantially retained in the third solvent stream, or if entering the third electrophoresis separation membrane, being substantially prevented from passing through the third electrophoresis separation membrane;

(o) optionally, periodically stopping and reversing the electric potential to cause movement of biomolecules having a molecular mass greater than immunoglobulins having entered the third electrophoresis membrane to move back into the treated third solvent stream, wherein substantially not causing any immunoglobulins that has entered the fresh fourth solvent stream to re-enter treated third solvent stream;

(p) maintaining steps (n) and optional (o) until the desired amount of immunoglobulins have been moved to the fresh fourth downstream;

5. The method according to claim 1 wherein phase IV comprises the steps:

(q) placing the albumin/ α -1-antitrypsin concentrate in a fifth solvent stream, the fifth solvent stream being separated from a sixth solvent stream by a fourth electrophoretic separation membrane having a molecular mass cut-off less than the molecular mass of albumin;

(r) selecting a buffer for the fifth solvent stream having a pH greater than neutral;

(s) applying an electric potential between the fifth and sixth solvent streams causing movement of α -1-antitrypsin through the fourth electrophoresis separation membrane into the sixth solvent stream while albumin is substantially retained in the fifth solvent stream, or if entering the fourth electrophoresis separation membrane, being substantially prevented from passing through the fourth electrophoresis separation membrane;

(t) optionally, periodically stopping and reversing the electric potential to cause movement of albumin having entered the fourth electrophoresis separation membrane to move back into the fifth solvent stream, wherein substantially not causing any α -1-antitrypsin that has entered the sixth solvent stream to re-enter the fifth solvent stream; and

(u) maintaining steps (s) and optionally (t) until the desired amount of albumin remains in the fifth solvent stream and the desired amount of α -1-antitrypsin has have been removed to the sixth solvent stream.

6. The method according to claim 1, 2 or 5 wherein phase IV is carried out after phase I.

7. The method according to any one of claims 1 to 6 wherein the plasma is a pooled human plasma sample.

8. The method according to claim 2 wherein the first electrophoresis separation membrane of step (a) has molecular mass cut-off of about 75 kDa and the restriction membrane has a molecular mass cut off of about 50 kDa.

9. The method according to claim 2 wherein the buffer in step (b) has a pH of 9.

10. The method according to claim 9 wherein the buffer is a Tris-borate buffer.

11. The method according to claim 3 wherein the second electrophoresis separation membrane of step (f) has a molecular mass cut-off of 200 kDa.

12. The method according to claim 3 wherein the buffer of the third solvent stream in step (g) has a pH of 9.

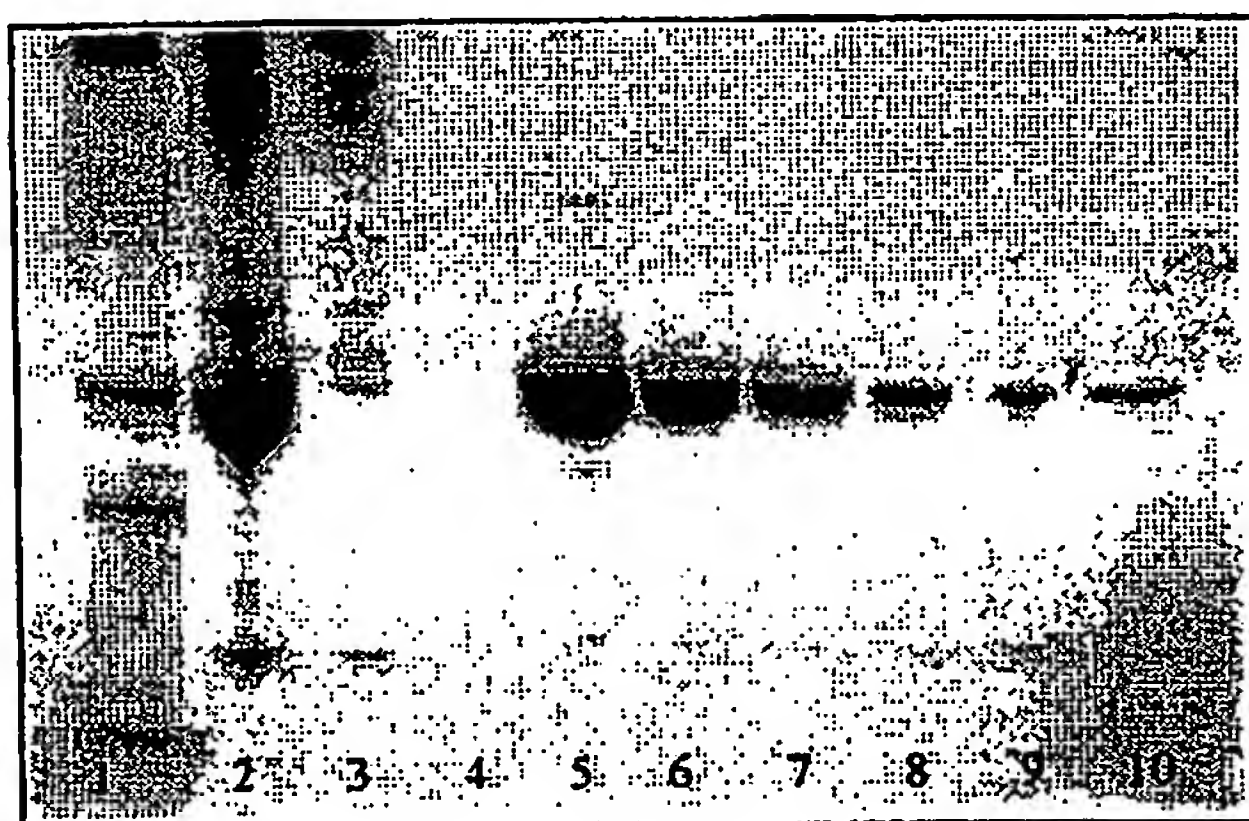
13. The method according to claim 4 wherein the third electrophoresis separation membrane of step (l) has a molecular mass cut-off of 500 kDa.

14. The method according to claim 4 wherein the buffer of the immunoglobulins concentrate of step (m) has a pH of less than 5.

15. The method according to claim 14 wherein buffer has a of pH 4.6.

16. The method according to claim 5 wherein the fourth electrophoresis separation membrane of step (q) has molecular mass cut-off of about 50 kDa.
17. The method according to claim 5 wherein the buffer of the fifth solvent stream in step (r) has a pH of 8.0.
- 5 18. The method according to claim 17 wherein the buffer is a Tris-borate buffer.
19. The method according to claim 2 wherein an electric potential of 250 volts is applied in step (c).
20. The method according to claim 3 wherein an electric potential of 250
10 volts is applied in step (h).
21. The method according to claim 4 wherein an electric potential of 250 volts is applied in step (n).
22. The method according to claim 5 wherein an electric potential of 250 volts is applied in step (s).
- 15 23. The method according to any one of claims 1 to 22 wherein the immunoglobulins are immunoglobulin G (IgG).
24. The method according to any one of claims 1 to 23 wherein yields of albumin, immunoglobulins and α -1-antitrypsin from plasma are at least 70% and purity of at least 90%.
- 20 25. The method according to any one of claims 1 to 24 wherein albumin, immunoglobulins and α -1-antitrypsin are separated from plasma in less than 1 day.
26. The method according to claim 25 wherein albumin, immunoglobulins and α -1-antitrypsin are separated from plasma in less than 12 hours.
- 25 27. The method according to claim 25 wherein albumin, immunoglobulins and α -1-antitrypsin are separated from plasma in less than 6 hours.
28. Use of Gradiflow™ technology in the purification and/or separation of albumin, immunoglobulins and α -1-antitrypsin from a pooled plasma sample.
29. The use according to claim 28 wherein the immunoglobulins are
30 immunoglobulin G (IgG).
30. Isolated albumin, immunoglobulins, and α -1-antitrypsin purified by the method according to any one of claims 1 to 27.
31. Isolated immunoglobulins according to claim 30 comprising immunoglobulin G (IgG).
- 35 32. Use of albumin, immunoglobulins and α -1-antitrypsin according to claim 30 in medical and veterinary applications.

Figure 1: SDS PAGE analysis of Albumin isolation



- 1: Molecular Weight Markers
- 2: Plasma
- 3: Upstream Residual
- 4: Downstream Time Zero
- 5-10: Downstream Albumin Product

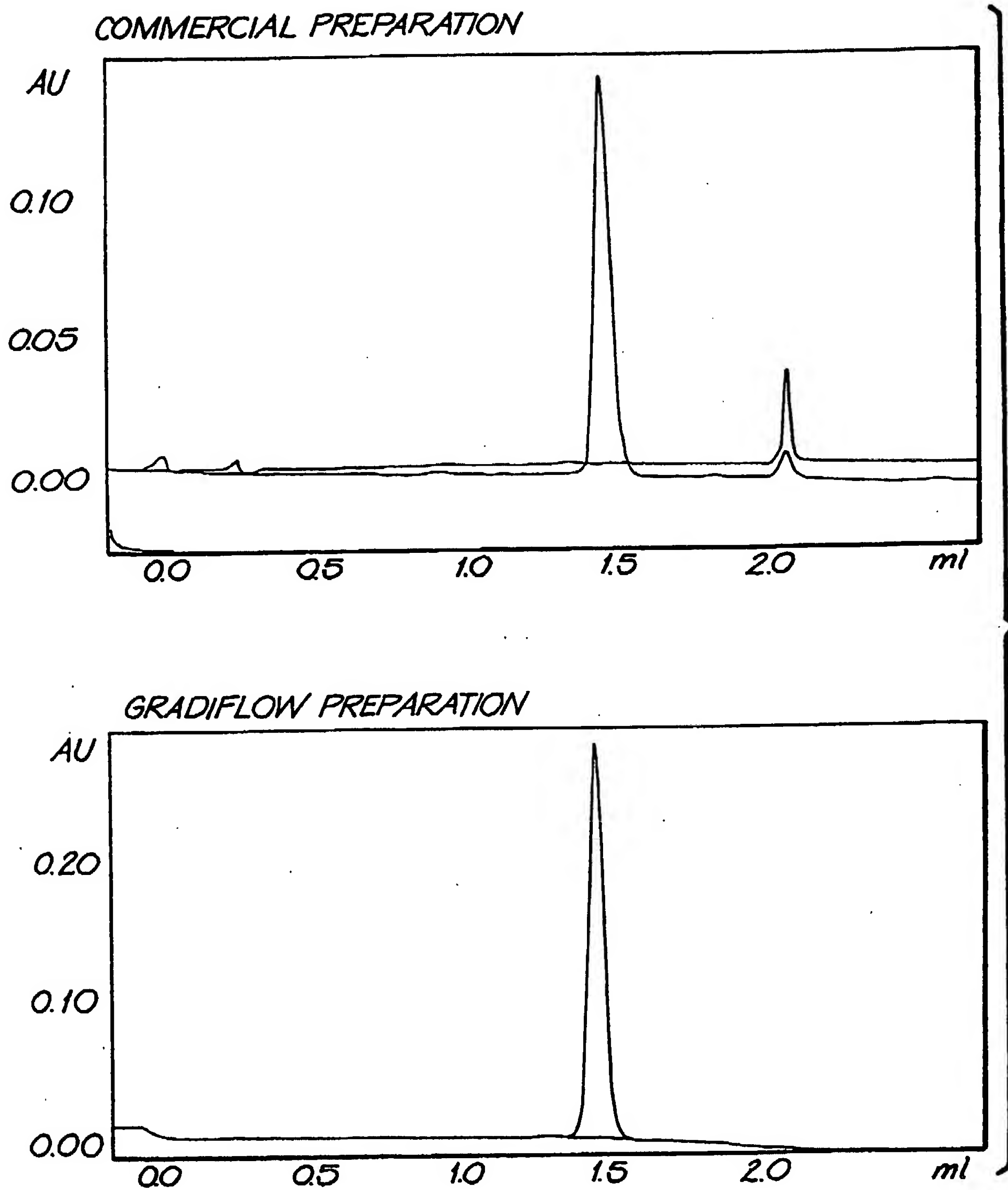
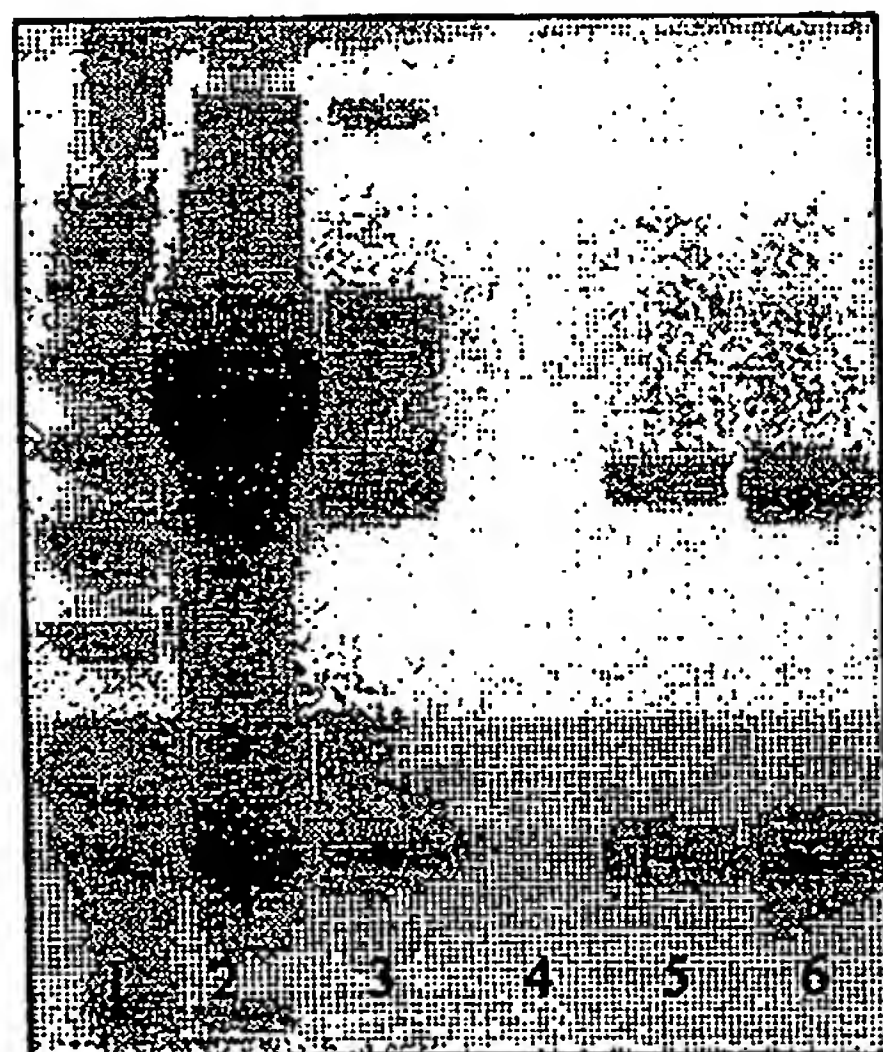
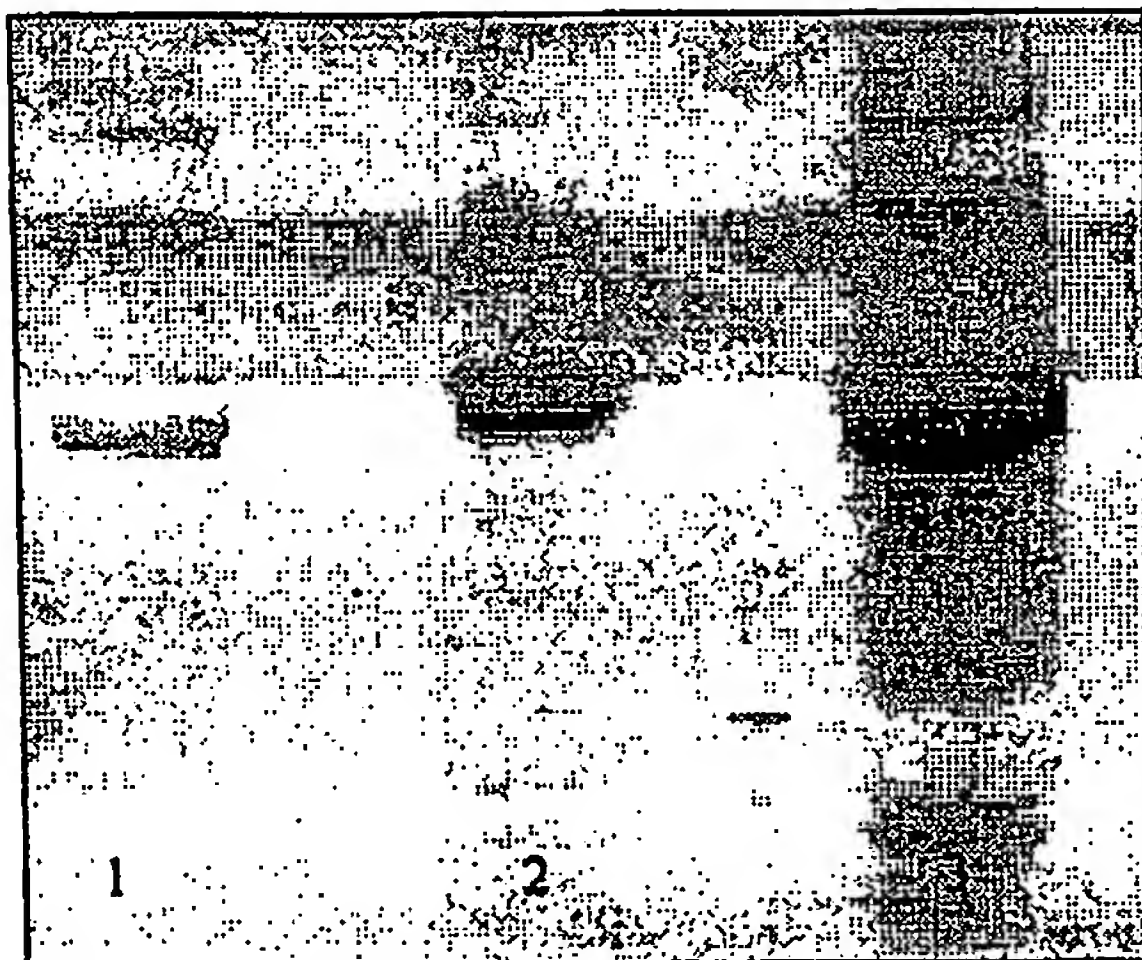
**FIG. 2**

Figure 3: Reduced SDS PAGE analysis of IgG isolation.



- 1: Markers
- 2: Plasma
- 3: Upstream Phase 1
- 4: IgG product- 30min
- 5: IgG product- 60min
- 6: IgG product- 90min

Figure 4: Western blot analysis of IgG isolation.



- 1: Plasma
- 2: Gradiflow IgG Product
- 3: Commercial IgG Preparation

5/8

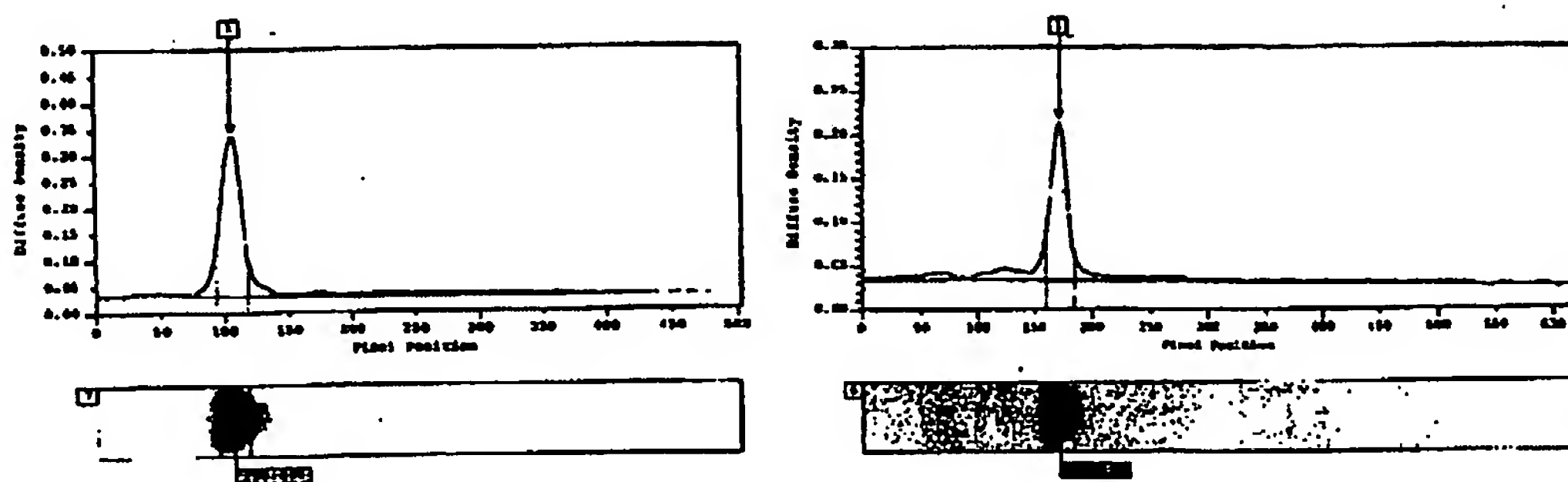
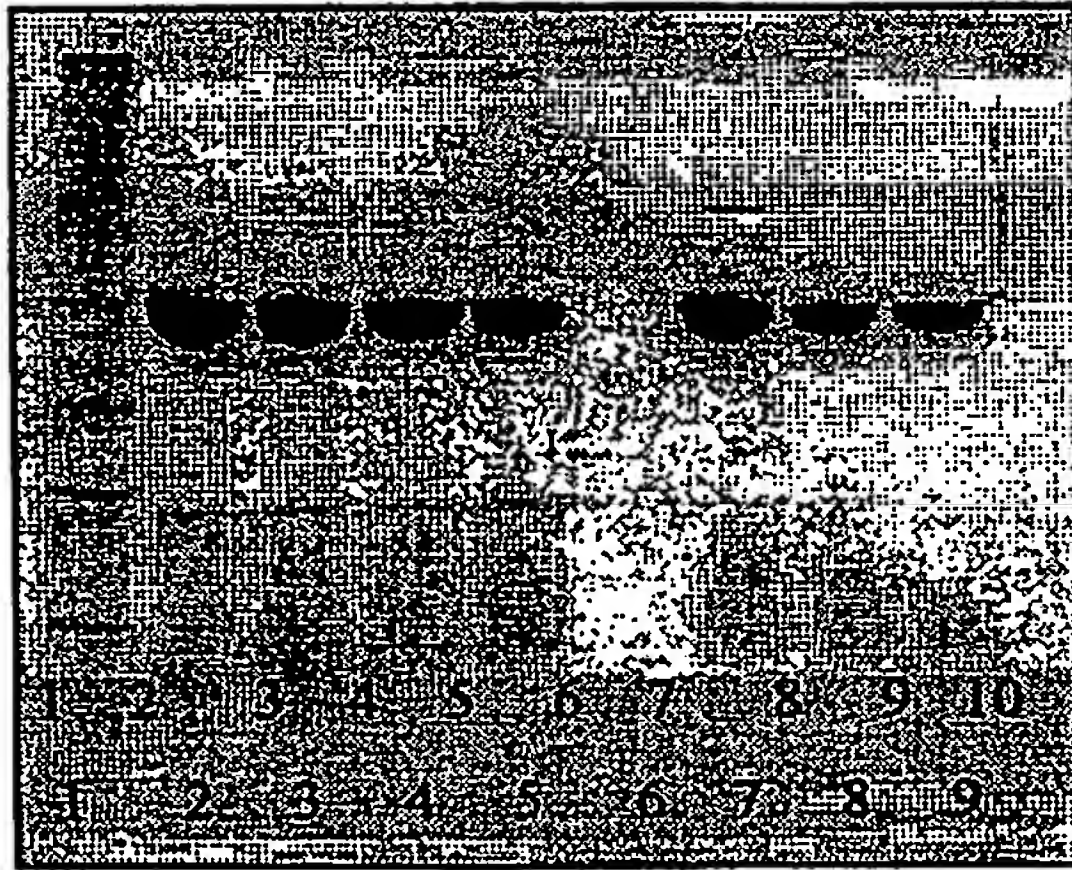
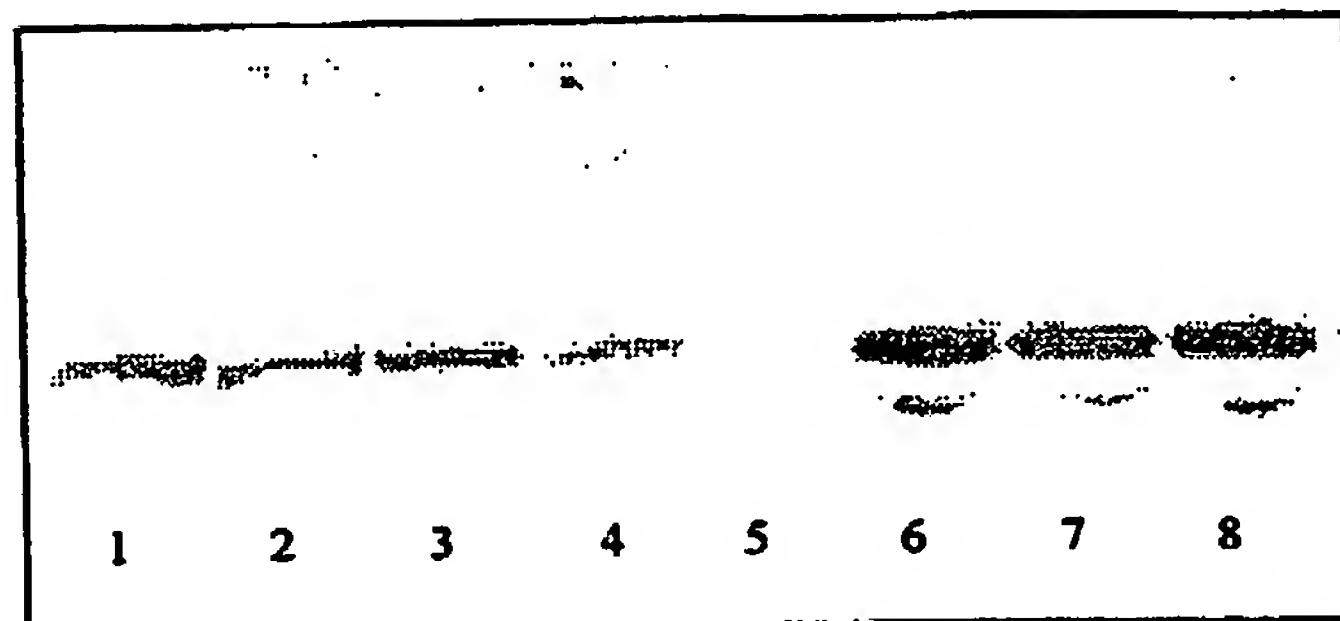
Figure 5: Non-reduced SDS PAGE phoretix of IgG isolation.**Commercial Preparation****Gradiflow Preparation**

Figure 6: Non-reduced SDS PAGE of α -1-antitrypsin isolation.



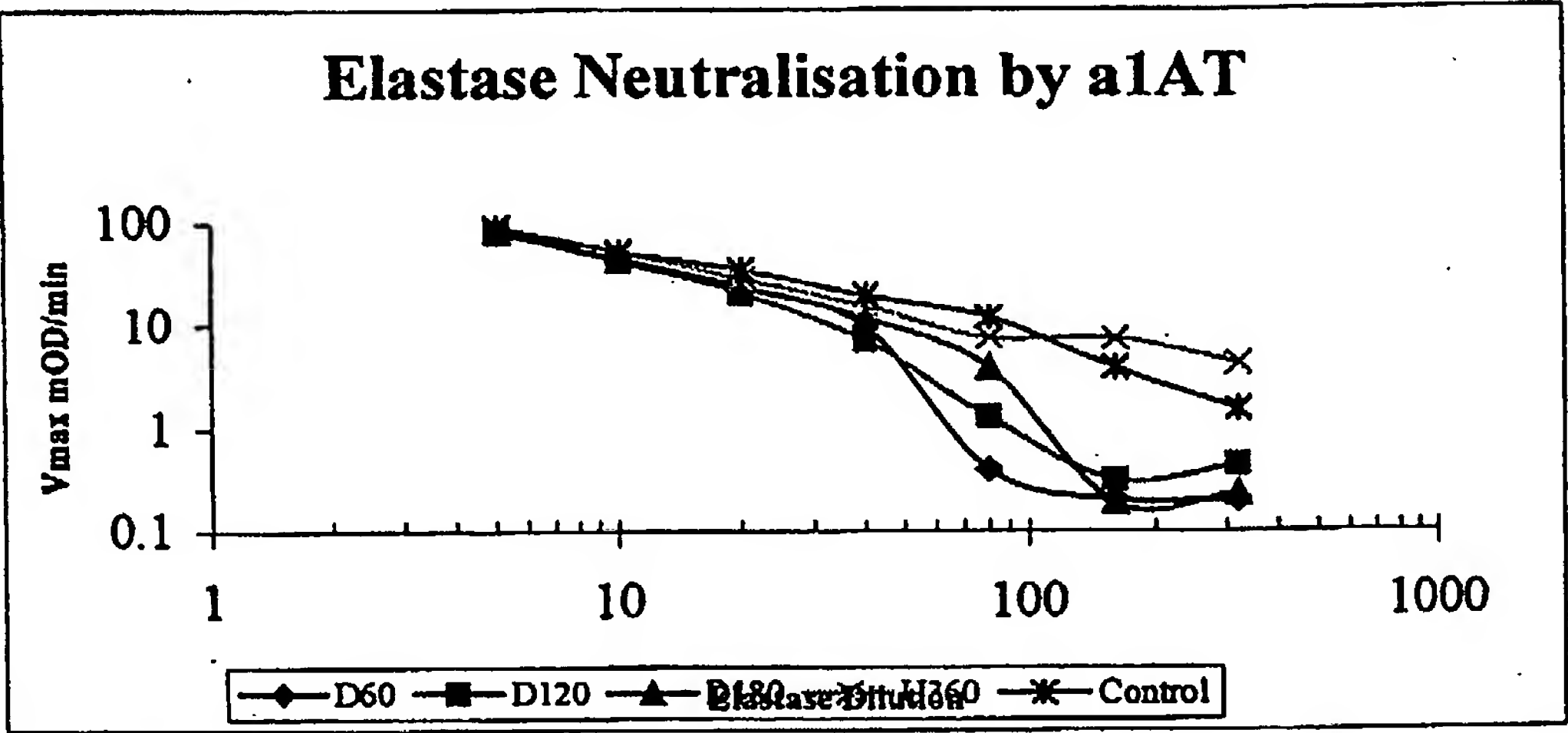
Lane 1: Molecular Weight Markers
 Lane 2: Gradiflow Albumin Product
 Lane 3-5: Upstream 1,2 and 3 hours
 Lane 6: Running Buffer
 Lane 7-9: α -1-antitrypsin products

Figure 7: Western analysis of non-reduced SDS PAGE of α -1-antitrypsin isolation.



1: Plasma
 2-4: Upstream 1, 2 and 3 hours.
 5: Downstream zero
 6-8: α -1-antitrypsin product

Figure 8: Functional analysis of Gradiflow isolated α -1-antitrypsin.



INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/00308

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. ⁷: C07K 1/26, C07K 14/47, C07K 14/76, C07K 16/06, C07K 16/34, B01D 71/74

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN: File WPID, File medline, File biosis, File HCA; Keywords: electrophor, plasma, albumin, anti-trypsin, antibod, immunoglob, membran,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 3,989,613 (Gritzner) 2 November 1976 See whole document, in particular column 1 line 39 to column 2 line 8.	1-33
A	EP, A2, 052 391 (Centre National de la Recherche Scientifique) 26 May 1982 See whole document	1-33
A	Journal of Chromatography A 827 (1998) 329-335. "Purification of monoclonal antibodies from ascitic fluid using preparative electrophoresis." See whole document.	1-33

☒ Further documents are listed in the continuation of Box C ☒ See patent family annex

* Special categories of cited documents:

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 "E" earlier application or patent but published on or after the international filing date
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 "O" document referring to an oral disclosure, use, exhibition or other means
 "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
 "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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 "&" document member of the same patent family

Date of the actual completion of the international search

15 June 2000

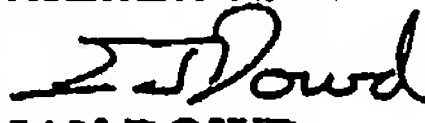
Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/00308

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Electrophoresis 1994, 15, 968-971. "Multifunctional apparatus for electrokinetic processing of proteins". See whole document.	1-33
A	Derwent Abstract Accession No. 85-041569/07, Class B04, JP 60-001134 A (FUJI REBIO KK) 7 January 1985	1-33
A	Derwent Abstract Accession No. 87-253908/36, Class S03, JP 62-175498 A (NITTO ELECTRIC IND KK) 1 August 1987	1-33
A	International Workshop of the University of Munich and the International Society for Artificial Organs. Rottach-Egern (FRG), March 17-19, 1983. "Plasma Separation and Plasma Fractionation. Current Status and Future Directions" Editors: M.J. Lysaght and H.J. Gurland, Munich	1-33

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/00308

Box I Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos :
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos : 28-29
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
No meaningful scope could be placed upon the term "Gradiflow technology" and no meaningful method steps could be elucidated.

3. ☐ Claims Nos :
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU00/00308

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
US	3989613	US	4043895		
EP	52391	AR	224972	AT	8465
		AU	543271	BR	8107229
		DE	3164907	ES	507393
		IE	52276	MX	153338
		US	4437967	ZA	8107701
				AU	77194/81
				CA	1161789
				FR	2493725
				NZ	198902

END OF ANNEX

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